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DOI: https://doi.org/10.1016/j.jid.2017.06.027

Accepted Manuscript

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PII: S0022-202X(17)32732-X

DOI: 10.1016/j.jid.2017.06.027

Reference: JID 986

To appear in: *The Journal of Investigative Dermatology*

Received Date: 13 January 2017

Revised Date: 30 May 2017

Accepted Date: 14 June 2017

Please cite this article as: Klar AS, Biedermann T, Michalak K, Michalczyk T, Meuli-Simmen C, Scherberich A, Meuli M, Reichmann E, Human adipose mesenchymal cells inhibit melanocyte differentiation and the pigmentation of human skin via increased expression of TGF-β1, *The Journal of Investigative Dermatology* (2017), doi: 10.1016/j.jid.2017.06.027.

Human adipose mesenchymal cells inhibit melanocyte differentiation and the pigmentation of human skin via increased expression of TGF-β1

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Keywords: skin tissue engineering, adipose mesenchymal cells, TGF- β1, melanogenesis, melanoblasts

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Abstract

There is accumulating evidence that interactions between epidermal melanocytes and stromal cells play an important role in the regulation of skin pigmentation. In this study we established a pigmented dermo-epidermal skin model (melDESS) of human origin to investigate the effects of distinct stromal cells on melanogenesis. melDESS is a complex, clinically relevant skin equivalent composed of an epidermis containing both melanocytes and keratinocytes. Its dermal compartment consisted either of adipose tissue-derived stromal cells (ASC), dermal fibroblasts, or a mixture of both cell types. These skin substitutes were transplanted for five weeks on the backs of immuno-incompetent rats and analyzed.

Gene expression and western blot analyses showed a significantly higher expression of transforming growth factor-β1 (TGF-β1) by ASC in comparison to dermal fibroblasts. In addition we showed that melanocytes responded to the increased levels of TGF-β1 by down-regulating the expression of key melanogenic enzymes such as tyrosinase. This caused decreased melanin synthesis and consequently greatly reduced pigmentation of melDESS.

The conclusions are of utmost clinical relevance, namely that that ASC derived from the hypodermis fail to appropriately interact with epidermal melanocytes thus preventing the sustainable restoration of the patient's native skin color in bio-engineered skin grafts.

Introduction

Human epidermal melanocytes are derived from the neural crest and reside in the stratum basale. They produce melanin - the pigment responsible for the skin color, package melanin into melanosomes, and transfer those to keratinocytes. The basic mechanism for melanin pigmentation is multicellular in design, consisting of a melanocyte and an associated population of keratinocytes, the functional epidermal melanin unit (EMU) (Ando et al., 2012). Melanin is stored within specific cytosolic organelles called melanosomes. Further, melanosomes can be transferred from melanocytes to epidermal keratinocytes to provide protection against ultraviolet (UV) light induced DNA damage, hence decrease the risk of mutations and skin cancer (Hearing et al., 1999).

In general, melanin biosynthesis is regulated by at least three specific enzymes, namely tyrosinase (TYR), tyrosinase-related protein 1 (TRP1) (Kobayashi et al., 1994), and tyrosinase-related protein 2 (TRP2; DCT) (Yokoyama et al., 1994). Among these enzymes, tyrosinase is the key enzyme catalyzing the rate-limiting step in melanin biosynthesis. The tyrosinase-related genes -TRP1 and TRP2 are under the control of the master transcriptional regulator of melanocyte differentiation and development - MITF (Microphthalmia Transcription Factor) (Bertolotto et al., 1998).

Moreover, two members of the SRY-box (SOX)-containing family of transcription factors play important roles in regulating melanocyte development (Harris et al., 2010). SOX9 has been implicated in melanocyte differentiation in the adult, whereas SOX10 is essential for the maintenance, migration, and specification of neural crest cells (NCCs) during embryogenesis (Cook et al., 2005). SOX9 is up-regulated by UVB exposure. In melanocytes, SOX9 increases the expression of MITF, TRP2, and tyrosinase promoters, leading to a stimulation of pigmentation (Passeron et al., 2007). In contrast, the high expression of SOX10 in unpigmented precursor cells is down-regulated as they differentiate into melanocytes (Cook et al., 2003, Greenhill et al., 2011, Harris et al., 2013). However, SOX10 expression is never fully abolished in differentiated melanocytes. It may still persist at lower levels in the adult, as SOX10 has also an important function at later melanocyte differentiation stages by activating MITF and TYR expression (Huber et al., 2003). However, the exact interplay between those different transcription factors and their impact on the maintenance of premature human melanocytes (melanoblasts) and their differentiation in human skin have not yet been fully characterized.

Increasing evidence suggests that, in addition to keratinocytes, stromal cells present in the human dermis are significantly involved in the regulation of skin pigmentation (Biedermann et al., 2015b). Yamaguchi *et al.* suggested that Dickkopf 1 (DKK1), an inhibitor of the Wnt signaling pathway produced by fibroblasts in the palms and soles, has an inhibitory effect on melanocyte proliferation and function, leading to a hypopigmentation of these body sites (Yamaguchi et al., 2008b, Yamaguchi et al., 2004).

We have recently successfully used adipose-derived mesenchymal stromal cells (ASC) to generate dermo-epidermal skin substitutes (Klar et al., 2014). ASC were shown to secrete different growth factors and cytokines, which improve wound healing by a paracrine mechanism (Kim et al., 2008a, Kim et al., 2008b) and show anti-oxidant effects (Kim et al., 2009a). Transforming growth factor-β1 (TGF-β1), one of the factors detected in the conditioned medium from ASC (ASC-CM), was shown in 2D cultures to inhibit melanin biosynthesis in B16 melanoma cells (Kim et al., 2008a, Martinez-Esparza et al., 1997). TGF-β1 regulates transcription (Shi and Massague et al., 2003) of various target genes such as MITF (Kim et al., 2004, Nishimura et al., 2010). Further, the reduced expression of MITF - the master regulator in melanocytes, inhibits the expression of its downstream effectors such as TYR and TRP1.

Although ASC were shown to be a promising cell source for skin equivalents (Klar et al., 2014), there have been no reports about the direct effects of ASC on melanocytes in a 3D *in vivo* skin model. Therefore, we studied the influence of ASC on melanogenesis in a pigmented bio-engineered dermo-epidermal skin model (melDESS) *in vivo*. We found that ASC suppressed melanocyte differentiation and skin pigmentation via inhibited melanin production by increased secretion of TGF- β1.

Results

The effect of the human ASC secretome on melanin production and proliferation of human epidermal melanocytes

The effect of human ASC-conditioned medium (ASC-CM) on the melanogenic activities of primary epidermal melanocytes was tested. When cultured in standard melanocyte growth medium (herein referred to as melanocyte medium) on culture plastic, melanocytes expressed PMEL. PMEL (PMEL17/gp100/premelanosome protein) is an amyloid fibril forming protein, specifically expressed in melanosomes and detectable by a HMB45 antibody (Raposo et al., 2001).

Under these culture conditions almost all PMEL+ cells showed the expression of tyrosinase (TYR) (94 ± 3%) (Figure 1a,g, Figure S1a-c), tyrosinase-related protein 1 (TRP1) (97 ± 5%) (Figure 1b,g, Figure S1d-f), and tyrosinase-related protein 2 (TRP2) (97 ± 3 %) (Figure 1c,g, Figure S1g-i).

Treating these melanocytes with ASC-CM for 72 hours markedly decreased the expression of TYR (8 ± 3%; $p<0.0001$) (Figure 1d,g; S1j-l) and TRP1 (9 ± 2%, $p<0.0001$) (Figure 1e,g, S1m-o), whereas it only marginally reduced the TRP2 (81 ± 6%; $p=0.0111$) (Figure 1f,g; S1p-s) expression in the PMEL+ melanocytes. Of note, the intensity of PMEL expression did not change in melanocytes upon addition of ASC-CM. However, the number of melanocytes was reduced by this treatment.

Moreover, melanocytes grown in ASC-CM showed lower cell numbers than control melanocytes cultured in melanocyte medium (Figure 1d-f; S1a-s). ASC-CM treated melanocytes exhibited small and bipolar cell bodies with a reduced number of dendrites, which is characteristic of melanocyte precursors (Nishimura et al., 2010). Visual inspection of the color of melanocyte pellets confirmed that ASC-CM caused a noticeable decrease of melanin content as compared to the control (Figure 1h).

Melanin content in melanocytes treated with ASC-CM was determined by a biochemical melanin assay (Figure 1h,i) (Bilodeau et al., 2001). Melanocytes cultured in ASC-CM showed significantly lower melanin content (14 ± 12 µg) as compared to those cultured in melanocyte medium (65 ± 11 µg). However, the downregulation of melanin synthesis by ASC-CM was almost fully reversed upon supplementing with melanocyte medium (47 ± 16 µg) (Kim et al., 2008a).

Comparison of factors secreted by human ASC and dermal fibroblasts

We have screened the expression of different cytokines such as DKK1, tumor necrosis factor (TNF)-α, interleukin (IL)-6, and Agouti-signaling protein (ASIP; MC1R antagonists) in both ASCs and dermal fibroblasts (Figure S2). Those factors were described in the literature as paracrine inhibitors of human melanocyte proliferation, tyrosinase activity, and

melanogenesis in human skin (Yamaguchi and Hearing et al., 2009; Wolf Horrell et al., 2016)). Notably, we did not observe a statistically significant difference in the expression of DKK1, IL-6, and ASIP when comparing ASCs and normal dermal fibroblast (not palmoplantar fibroblasts as reported by Yamaguchi et al., 2008b) (Figure S2). We observed a moderate upregulation of TNF-α mRNA in ASCs (Figure S2). Nevertheless, it was reported previously that the concentration of TNF-α in ASC-CM is much lower than its half-maximal inhibitory concentration (IC50) for having an inhibitory effect on melanocyte proliferation and melanogenesis (Kilroy et al., 2007, Kim et al., 2008a, Kim et al., 2008b).

Finally we observed a highly upregulated TGF-β1 mRNA and protein expression in ASC as compared to dermal fibroblast (Figure 2). Therefore, we concluded that TGF-β1 mediates most of the anti-melanogenic effects of ASC-CM in this study.

Figure 2a demonstrates TGF-β1-specific mRNA as shown by a band of the correct molecular weight. TGF-β1 mRNA was thus produced by both ASC and dermal Fb. However, TGF-β1 mRNA expression was almost 3-fold higher in ASC than in dermal Fb ($p=0.0037$) (Figure 2b).

Similar results were obtained analyzing protein levels. Western blot analyses revealed a 3-fold higher expression of TGF-β1 in ASC-cell lysates when compared to dermal Fb (Figure 2c).

Interestingly, ELISA measurements also revealed a significant difference in the concentration of TGF-β1 in conditioned media of both cell types (Figure 2d). TGF-β1 concentrations in ASC-CM were 2.4-fold higher than in dermal Fb-CM (200 ± 25 pg/ml and 84 ± 40 pg/ml; $p=0.0028$, respectively).

Skin pigmentation in human dermo-epidermal skin substitutes is regulated by stromal cells

We previously reported that bio-engineered pigmented dermo-epidermal skin substitutes (melDESS) composed of dermal fibroblasts (dermal compartment) and keratinocytes with melanocytes (epidermal compartment) from either dark or light donor skin, resulted in a

donor skin-matched dark or light coloration of those skin grafts, respectively (Bottcher-Haberzeth et al., 2013).

In this study, we investigated the influence of human dermal FB and ASC on pigmentation in melDESS in vivo. A hypothetical view comparing these two different models in vivo is depicted in Figure 7. From this model, it is evident that those two different types of stromal cells cause different effects on epidermal melanocytes in bio-engineered skin equivalents.

melDESS were transplanted on the backs of immune-deficient ralents.ts and excised after five weeks for analysis (Figure 3). Human dermal fibroblasts derived from dark pigmented donor skin were used in control skin substitutes (Figure 3a). We also included in the study melDESS containing a mixture of dermal Fb and ASC (dermal Fb/ASC) in a 1:1 ratio (Figure 3b). The epidermis contained the same keratinocytes and melanocytes isolated from dark pigmented donor skin in all three skin substitutes. Thus, solely the impact of the stromal cell component on melanocyte proliferation, skin pigmentation, and marker expression was investigated *in vivo*.

Five weeks after transplantation, the human skin substitutes containing dermal Fb showed intense dark pigmentation (Figure 3a), hence resembling the dark pigmented donor skin. Macroscopic evaluation of skin substitutes containing the mixed population of dermal Fb and ASC showed a slightly brown pigmented skin color (Figure 3b), whereas substitutes containing only ASC remained as a white, non-pigmented skin (Figure 3c). Skin pigmentation was homogenously distributed throughout the entire transplant in all groups.

Fontana Masson staining was undertaken to visualize melanin granules in the different skin substitutes. The staining demonstrated high melanin abundance in the basal layer and in supranuclear caps of keratinocytes in skin transplants containing dermal Fb (Figure 3d). In contrast, mixed transplants containing both Fb and ASC in the dermal part showed very low melanin content in basal epidermal cells and lacked the supranuclear keratinocyte melanin caps. Only some melanin granules were randomly distributed throughout all the epidermal keratinocyte layers (Figure 3e). Finally, in skin substitutes

containing ASC, melanin was only observed in few basal cells, whereas almost no melanin was found in suprabasal epidermal keratinocytes (Figure 3f).

MITF immunofluorescence of skin transplants revealed that melanocytes resided at the proper physiological location in the basal layer of the epidermis in all three skin specimens (Figure 3g-i). However, as demonstrated by the quantification in Figure 3j, the number of MITF-positive melanocytes was higher in the dermal Fb-group (18 ± 4) as compared the group containing a mixture of dermal Fb and ASC (12 ± 5, p=0.0058). Importantly, the presence of pure ASC in the dermal compartment of melDESS led to a statistically significant decrease in the mean melanocyte number in the epidermal basal layer (8 ± 3, p< 0.0001) (Figure 3j).

Decreased expression of TYR and TRP1 in ASC-containing melDESS *in vivo*

To assess cell density and melanocyte differentiation in transplanted skin substitutes, we performed combined immunostainings for PMEL, TYR, TRP1, and Ki67 (Figure 4a-f, S3a-d). The number of TYR^+PMEL^+ melanocytes in ASC-containing samples was significantly reduced as compared to skin substitutes containing dermal Fb (98 ± 5% vs. 11 ± 5%; p< 0.0001) (Figure 4a-c). Interestingly, melanocytes in skin transplants containing both dermal Fb and ASC expressed TYR at an intermediate level (60 ± 1% of all PMEL cells) (Figure S3a,c).

Additionally, TRP1, which is characteristic for late stage III or IV pigmented melanosomes, was expressed at a significantly lower level in ASC-containing specimens as compared to the dermal Fb-group (95 ± 10% vs. 3± 5%; p< 0.0001) (Figure 4d-f). In dermal Fb/ASC containing transplants the mean of 65 ± 5% of all PMEL+ melanocytes expressed TRP1 (Figure S3b,c). In contrast, TRP2 was expressed at comparable levels in all three transplant groups (data not shown). These finding suggest that ASC-CM blocks melanosome biogenesis in epidermal melanocytes at stage II.

Quantification of Ki67+ melanocytes in the ASC-group showed that only about 15 ± 7% of $PMEL^+$ melanocytes were proliferating as compared to 54 ± 20% in the control group

containing dermal Fb (Figure S3c-d) (p=0.0096). In skin transplants containing both Fb and ASC, a population of 43 ± 26% of proliferating melanocytes was detected (Figure S3c).

Differential expression of SOX proteins in melanocytes in ASC-containing melDESS *in vivo*

To further assess the differentiation status of melanocytes in the distinct skin substitutes, sections of skin transplants were stained with either SOX9 or SOX10 antibodies, which are characteristic for mature or immature melanocytes, respectively (Figure 4g-l).

Immunofluorescence analyses of SOX9 on skin transplants containing dermal Fb revealed that the vast majority of $PMEL^+$ melanocytes were positive for this differentiation marker (95 ± 7% of $PMEL^+$ cells) (Figure 4g,j). This finding is line with data we observed in normal human skin (data not shown). In contrast, melDESS containing ASC showed a significant reduction of SOX9 expression (40 ± 1% of $PMEL^+$ cells) (Figure 4h,i). Expression levels of SOX9 in skin transplants containing both Fb and ASC were similar to the dermal Fb-group accounting for 89 ± 5% of $Sox9^+PMEL^+$ cells (Figure S4).

Using a SOX10-specific antibody we observed just the opposite result as demonstrated in Figure 4j-l. Whereas only 39 ± 14% of melanocytes in the dermal Fb-group were SOX10-positive (Figure 4j,l), its expression increased to 81 ± 13% in the ASC-containing melDESS (Figure 4k,l). The expression level of SOX10 in skin transplants containing both stromal cells (dermal Fb and ASC) was reduced and accounted for 68 ± 13% of all melanocytes (Figure S4).

The effect of TGF-β1 on cultured human melanocytes

To examine the possible effect of TGF-β1 on human melanocytes *in vitro*, we tested its impact on melanocyte proliferation, morphology and differentiation *in vitro*.

Treatment of human epidermal melanocytes with synthetic TGF-β1 (2ng/ml) for 72 hours *in vitro* reduced the expression level of TYR from 92 ± 3% to 33 ± 6% (p<0.0001) (Figure 5a, d, g) (Martinez-Esparza et al., 1997). The expression of TRP1 was also reduced

from 90 ± 3% to 29 ± 2% ($p<0.0001$) (Figure 5b,e,g) (Martinez-Esparza et al., 1997) as compared to control cells cultured in melanocyte medium. However, treatment with TGF-β1 did not significantly alter the expression of TRP2: 98 ± 2% vs. 95 ± 4% ($p=0.2926$) (Figure 5c,f,g).

In addition we found that the overall number of melanocytes appeared lower in the TGF-β1 treated group (88 ± 8 vs. 19 ± 3 cells/field; $p=0.0002$) (Figure 5h). We also observed morphological changes elicited by TGF-β1 (Figure 5d-f). Whereas untreated melanocytes exhibited a pronounced cell body and multiple dendrites, the TGF-β1 treated melanocytes appeared mostly as spindle shaped, bipolar cells exhibiting elongated dendrites.

Discussion

Skin pigmentation is a central issue in the production of bio-engineered, autologous clinically applicable dermo-epidermal skin substitutes. To study pigmentation in those substitutes in a close to physiological context, we investigated the impact of distinct stromal cell types on skin color. Given the fact that ASC are derived from the subcutis, we asked whether these cells that physiologically do not directly interact with the epidermis, would support epidermal pigmentation efficiently and in all aspects. This was of particular interest as ASC represent an abundant cell source and can be used as a substitute for dermal fibroblasts, especially if the latter are limited in number. To gain more insight into this matter, we bio-engineered pigmented dermo-epidermal skin substitutes (melDESS) containing epidermal melanocytes and keratinocytes, and differing in the distinct types of stromal cells in the dermal part (Biedermann et al., 2015b). The overall results revealed an anti-melanogenic effect of ASC. This effect can be explained by a significant inhibition of melanocyte proliferation, differentiation, and melanogenesis.

We demonstrated that trophic factors secreted by ASC, in particular TGF-β1, maintain melanocytes in an immature, progenitor-like state, thus inhibiting melanin synthesis. Accordingly, we observed a strong down-regulation of TYR and TRP1 in human melanocytes treated *in vitro* with medium containing synthetic TGF-β1 (Martinez-Esparza et al., 1997).

These findings may well point to a paracrine mechanism induced, perhaps not exclusively, but at least to a significant degree, by an increased TGF-β1 concentration.

Further, we cannot exclude that other growth factors or cytokines secreted by ASC such as DKK1, TNF-α, IL-6, or ASIP, which are known inhibitors of melanin synthesis and melanosome transfer (Kim et al., 2008a), might exert a combinatorial effect on melanocytes. Nevertheless, the concentration of those factors in ASC-CM is not high enough to play a crucial role in in the whitening of skin transplants in this study (Kilroy et al., 2007, Kim et al., 2008a, Martinez-Esparza et al., 1997, Martinez-Esparza et al., 1998).

Interestingly, already the macroscopic evaluation of the skin transplants containing ASC revealed massively reduced skin pigmentation in vivo, resulting in an almost non-pigmented “white” epidermis. This phenomenon was partially compensated by mixing dermal fibroblasts with ASC (in a ratio of 1:1). The key enzymes involved in melanin synthesis – TYR and TRP1, were significantly reduced in melDESS containing ASC. Similar findings showing a suppression of TYR and TRP1 after the treatment with ASC-CM were described by Kim *et al*. in B16 mouse melanoma cells, growing on cell culture plastic, hence in a 2D *in vitro* setting (Kim et al., 2008a).

In human skin epidermal melanocytes exist in distinct maturation states. We and others have demonstrated that SOX9 is expressed in a subpopulation of melanocytes and that this correlates with the presence of MelanA$^+$ and PMEL$^+$ mature cells in the epidermis and in the hair matrix of normal (non-palmoplantar) human skin (Biedermann et al., 2015a, Krahl and Sellheyer et al., 2010). In contrast, SOX10 plays an important role during the early development of melanocytes in the neural crest, and is still expressed in immature melanocytes in the epidermis (Greenhill et al., 2011, Harris et al., 2013). However, SOX10 expression was detected only in approximately 1 of 6 melanocytes in normal human skin (Hasegawa et al., 2008). In our study, the presence of ASC significantly decreased the proportion of SOX9-positive melanocytes in melDESS, whereas the fraction of immature SOX10 expressing melanocytes was significantly increased. Interestingly, Cook *et al.* demonstrated that cultured immature melanoblasts derived from human neonatal foreskin

also expressed SOX10 at high levels, which decreases as these cells differentiate into mature melanocytes (Cook et al., 2003).

In conclusion it can be stated that the mesenchyme (the stroma) matters when it comes to the regulation of epidermal melanocyte development in bio-engineered skin (see also Yamaguchi et al., 2005). In addition it is the cellular composition of the dermal stroma which determines the ratio of mature and immature melanocytes, hence their capability to produce, process, and transfer melanin to keratinocytes (Plonka et al., 2009, Yamaguchi and Hearing et al., 2009). We have demonstrated here that TGF-β1 plays a central role in this regulation.

Previously we have shown that the composition and properties of a particular stroma also varies significantly with respect to its body site (Biedermann et al., 2015b). As a consequence we need to consider both, from which dermal layer the stromal cells are derived, and from which area of the body stromal cells are isolated, to bio-engineer a skin graft that optimally matches the color of the particular skin region. As a consequence of these insights and conclusions, we would like to expand the current concept of the Epidermal Melanin Unit (EMU) to the novel concept of the Dermo-Epidermal Melanin Unit (DEMU). The DEMU consists of a given melanocyte that is guided not only by keratinocytes but also by underlying stromal cells.

Materials and Methods

Human skin and adipose tissue samples

The study was conducted according to the “Declaration of Helsinki Principles” and after permission by the ethic commission of the Canton Zurich. Parents gave informed consent to use skin samples. The human foreskins used were obtained from dark skin pigmentation types from patients 1 to 16 years of age.

Human subcutaneous adipose tissue samples were obtained from fat excisions from healthy human donors (between 18 and 68 years of age), female or male, mostly from abdominal body location, all of them undergoing a surgical excision operation. All donors

gave their informed consent and the study protocol was approved by the local ethical committee (EKBB, Ref. 78/07 and 2006-192NMA [extended in 2009]).

Isolation and culturing of primary cells

The dark pigmented skin samples were used for the isolation of human epidermal melanocytes, keratinocytes, and dermal fibroblasts as previously described by Bottcher-Haberzeth et al., 2013).

Adipose stromal cells (ASC) were obtained after a monolayer expansion of freshly isolated stromal vascular fraction (SVF) as previously described by Klar et al., 2014.

A more complete and detailed description of the methods is included in Supplementary Materials and Methods.

Conflict of interests

The authors declare no conflict of interest.

Acknowledgments

The authors would like to acknowledge Ms. Susanne Staubli for creating the schematic skin model in figure 6. This work was financially supported by the Clinical Research Priority Programs (KFSP: From basic research to the clinic: Novel tissue-engineered skin grafts for Zurich) of the Faculty of Medicine of the University of Zurich. The authors are particularly grateful to the Foundation Gaydoul for their generous financial support.

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Figure legends

Figure 1 Effect of ASC-CM on the expression of melanocyte markers and melanin content. (a-f) Human primary melanocytes were cultured in either melanocyte medium (a-c) or in ASC-CM (d-f). Melanocytes were stained for: PMEL (red), and TYR, or TRP1 or TRP2 (green). (g) When cultured in melanocyte medium, melanocytes expressed TYR (94±3.1%) (a,g), TRP1 (97±4.8%) (b,g), and TRP2 (97±2.5%) (c,g). In ASC-CM the expression of TYR (8±2.9%) (d,g) and TRP1 (9±2.4%) (e,g) was markedly reduced, whereas TRP2 expression was only slightly reduced (81±5.7%) (f,g). Blue: cell nuclei. Scale bars=50 μm. Error bars: mean +/- SD. (h) Melanocyte pellets after incubation in melanocyte medium (left), ASC-CM (middle) followed by melanocyte medium (right) for 1week. (i) The melanin content was extracted from melanocytes and measured by spectrophotometer at 405nm.

Figure 2 Analysis of TGF-β1 expression in dermal fibroblasts (Fb) and ASC. (a) Comparison of TGF-β1 mRNA expression in cultured dermal Fb and ASC by reverse transcription-PCR (RT-PCR) (green inset). GAPDH was used as an internal control. (b) Relative expression levels of TGF-β1 mRNA calculated as a normalized area of TGF-β1 peak. (c) Western blot analysis of TGF-β1 protein isolated from cell lysates of cultured dermal Fb and ASC. (d) ELISA of TGF-β1 protein isolated from supernatants of cultured dermal Fb and ASC. Error bars: mean +/- SD.

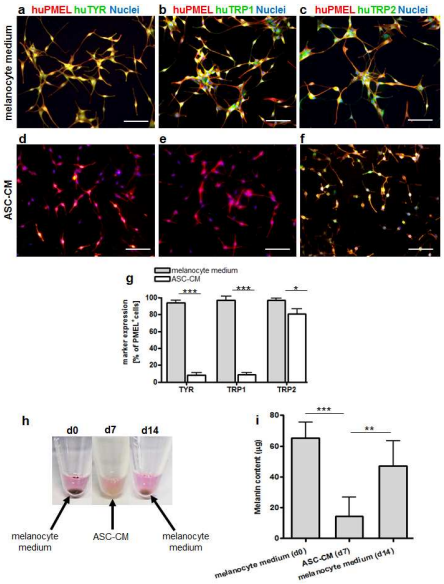
Figure 3 Assessment of skin pigmentation of different melDESS *in vivo.* (a-c) Macroscopic view of dermal Fb, dermal Fb/ASC, and ASC melDESS 5 weeks post-transplantation. (d-f) Fontana-Masson staining demonstrated the highest melanin concentration in basal layer cells of dermal Fb-constructs (d), whereas melanin was less abundant or absent in dermal Fb/ASC or ASC-constructs, respectively (e-f). Only the dermal Fb-group displayed melanin in supranuclear caps suprabasally (d, white arrows, inset), while other melDESS showed random or no suprabasal melanin distribution, respectively (e-f, white arrows, insets). White dotted line: basement membrane. (g-i) MITF-staining (red) confirmed even melanocyte (black arrows) distribution throughout all melDESS basal layers and revealed differences debetween dermal Fb (18±4), dermal Fb/ASC (12±5), and ASC (8±3) melDESS (j). Scale bars=50 µm, insets=10 µm. n=4, 24 rats.

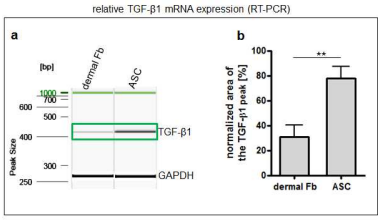
Figure 4 Expression of melanocyte differentiation markers in melDESS *in vivo.* (a-c) The number of $TYR^{+}PMEL^{+}$ (white arrows) melanocytes was significantly decreased (11±5% vs. 98±5%, $p<0.0001$) in ASC skin substitutes versus dermal Fb group. (d-f) Comparison of TRP1 expression of $PMEL^{+}$ melanocytes of dermal Fb (d) and ASC (e) skin substitutes. (g-i) SOX9, a marker of mature melanocytes, was rarely expressed in PMEL-positive melanocytes, when the skin substitute were constructed using ASC alone (40 ± 1%), and strongly expressed in the dermal Fb group (95±7%, $p<0.0001$). (j-l) A significantly higher number of $SOX10^{+}$ $PMEL^{+}$ immature melanocytes was detected in melDESS containing ASC

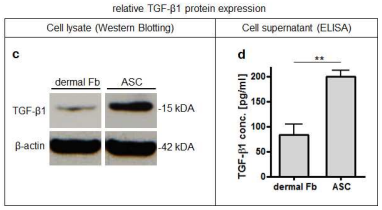
(81±13%) than dermal Fb (39±14%, p<0.0001). Scale bars=50µm, inset =10 µm. Error bars: mean +/- SD, n=4, 24 rats.

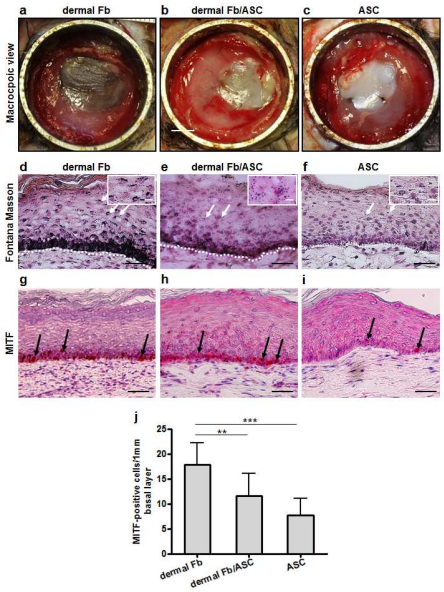
Figure 5 The impact of TGF-β1 on the marker expression in cultured melanocytes. (a-f) Human primary melanocytes were cultured either in melanocyte medium (a-c) or treated with 2ng/ml TGF-β1 (d-f) for 72 hours *in vitro.* (g) The TGF-β1 treatment significantly reduced the expression of TYR (33±6% vs. 92±3%, p<0.001) and TRP1 (29± 2% vs. 90±3%, p<0.001) in melanocytes. The expression of TRP2 was not significantly changed by the treatment (95±4%) as compared to control cells (98±2%, p: ns). (h) TGF-β1 reduced the cell density of melanocytes from 88±8 to 19±3 cells per field (p<0.001). Blue: cell nuclei. Error bars: mean +/- SD, n=3.

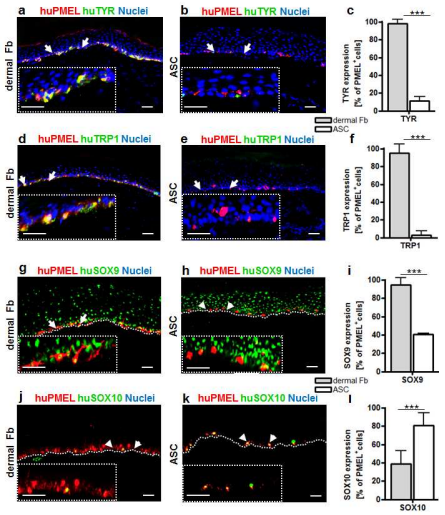
Figure 6 Hypothetical scheme showing stromal-epithelial interactions in two different melDESS models *in vivo.* (a) Control skin model constructed with dermal Fb, keratinocytes and melanocytes. Dermal Fb secrete low levels of TGF-β1 and support growth and differentiation of epidermal melanocytes. Hence, dermal Fb facilitate dark pigmentation of skin substitutes *in vivo.* (b) Skin model constructed with ASC, keratinocytes and melanocytes. ASC exhibit strong inhibitory effects on melanocytes in the epidermis due to a high secretion of TGF-β1. This cytokine decreases the pigmentation of skin substitutes *in vivo* by inhibiting the proliferation as well as TYR and TRP1 activity in melanocytes.

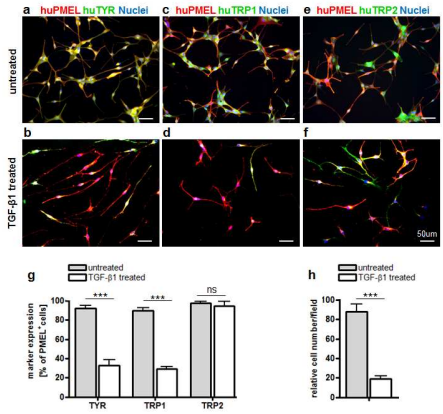






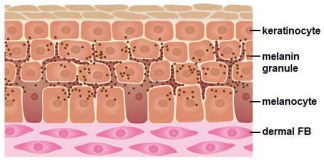






a

Skin model using black dermal fibroblasts (dermal FB), black keratinocytes and melanocytes



b

Skin model using adipose-derived mesenchymal cells (ASC), black keratinocytes and melanocytes